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Minireview

Dynamic control of TGF-β signaling and its links to the cytoskeleton

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Abstract Transforming growth factor β (TGF-β) regulates cellular behavior in embryonic and adult tissues. TGF-β binding to serine/threonine kinase receptors on the plasma membrane activates Smad molecules and additional signaling proteins that coordinately regulate gene expression or cytoplasmic processes such as cytoskeletal dynamics. In turn, the activity and duration of the Smad pathway seems to be regulated by cytoskeletal components, which facilitate the shuttling process that segregates Smad proteins in the cytoplasm and nucleus. We discuss mechanisms and models that aim at explaining the coordination between several components of the signaling network downstream of the TGF-β signal.

Keywords: Actin; BMP; Microtubule; Smad; Signal transduction; TGF-β; Transportin

1. Introduction

The transforming growth factor β (TGF-β) pathway plays important roles in regulating cell physiology during embryonic development and adult life, and perturbation of this pathway is linked to a plethora of human diseases [1]. Here, we focus on specific aspects of signal transduction regulation and on selected physiological outputs of TGF-β. We highlight the mechanisms and importance of nucleocytoplasmic shuttling of Smad molecules in maintaining the potency of TGF-β signaling, and we review links of this pathway with regulation of the cytoskeleton. These two aspects are currently investigated with an increasing pace.

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Abbreviations: ALK, activin receptor-like kinase; αSMA, α-smooth muscle actin; BMP, bone morphogenetic protein; Dpp, decapentaplegic; EMT, epithelial-mesenchymal transition; FGF, fibroblast growth factor; GDF, growth differentiation factor; GEF, guanine exchange factor; GSK3β, glycogen synthase kinase 3β; MAPK, mitogen-activated protein kinase; NES, nuclear export signal; NLS, nuclear localization signal; PI3K, phospho-inositide 3′-kinase; RTK, receptor tyrosine kinase; SBE, Smad-binding element; TAK1, TGF-β-activated kinase 1; TGF-β, transforming growth factor β

2. TGF-β ligands and their extracellular regulation

In humans, TGF-β represents a family of secreted polypeptides that include 33 distinct gene products, most of which form disulfide-bonded homodimers and some of which are also capable of forming heteromeric ligands [1]. Biosynthesis of TGF-β mRNA, its protein in the endoplasmic reticulum, its secretion through the Golgi apparatus and the exocytic vesicular system, and storage in the extracellular matrix, is a complex process that imparts several regulatory steps [2]. The matrix-stored form of latent TGF-β requires biochemical activation, via proteolytic cleavage, interaction with integrins and mechanochemical force, such as that generated by myofibroblast contraction, before recognizing its cognate cell surface receptors [3,4].

The TGF-β family, in addition to the three TGF-β members (TGF-β1, -β2, -β3), includes the bone morphogenetic proteins (BMP), many of which are also named growth differentiation factors (GDF), some unique GDFs, the anti-Müllerian hormone (AMH), the activins, inhibins, and nodals [1]. All these ligands signal via a small number of cell surface receptors and are involved in numerous physiological processes during embryogenesis and organ formation as well as tissue homeostasis in adults.

Especially during embryogenesis, but possibly during adult tissue homeostasis as well, the secreted TGF-β ligands travel certain distance from the producer cells to the responder cells, and thus form morphogenetic gradients [5,6]. Ligand gradients are regulated by complementary mechanisms, such as, cell-specific secretion across the tissue, gradients of antagonistic extracellular proteins that bind and inactivate the ligands, and gradients of proteases that degrade the extracellular antagonists and release bioactive ligands [7]. One model that explains the mechanics of ligand gradient formation proposes a mechanism of transcytosis in the wing imaginal disc of *Drosophila*, whereby the BMP-like ligand decapentaplegic (Dpp) is endocytosed by the first cell, without inducing its signaling pathway, and then exocytosed from the opposite side of the same cell in order to be engulfed by the neighboring adhering cell, and thus Dpp travels across the multicellular embryonic tissue in vesicles through the main cell body and via cell–cell junctions [8]. According to another model, in early mouse embryos, the secreted ligand Nodal travels along the basolateral cell surface, always in association with plasma membrane-tethered sulfated glycosaminoglycans, until it reaches its end target cell where signaling initiates germ layer specification and subsequent morphogenesis [9]. What defines the target cell that will initiate Dpp or Nodal signaling according to these two models remains currently unknown, but has been proposed to depend

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on nuclear factors that regulate the transcriptional activity of the transducers of the Dpp/Nodal signal, the Smad proteins, or their nuclear residence [10,11]. Currently, none of the above models explain this important question.

TGF-β family studies in developing embryos of *Drosophila*, *Xenopus* and the mouse, have emphasized the need for regulation of signal strength and duration [6]. This concept is not unique to TGF-β, and has been analyzed in more detail, e.g. in the mitogen activated protein kinase (MAPK) pathway downstream of various mitogenic factors [12]. However, the mechanics of how extracellular TGF-β signals are interpreted in a quantitative and time-dependent way remain largely unexplained. Significant recent advances in understanding such dynamic processes are underway [13].

3. Signaling via TGF-β receptors

The TGF-β family signals via the small family of receptor serine/threonine kinases that include 7 type I and 5 type II receptors [14]. All 12 receptors are single-pass transmembrane proteins with a short cysteine-rich extracellular, ligand-binding domain, an α-helical transmembrane domain and a long cytoplasmic domain that primarily consists of the kinase and secondarily of additional protein sequences that serve either as phospho-acceptor sites or as docking sites for interacting adaptor or signaling proteins. The 7 type I receptors are best known as activin receptor-like kinases 1 to 7 (ALK1–ALK7) and each of them serves the signaling needs of multiple ligands of the TGF-β family [14]. The 5 type II receptors are the two activin/BMP receptors ActRIIA, ActRIIB, the BMP receptor BMPRII, the TGF-β receptor TGFβRII and the AMH receptor AMHRII [14].

During biosynthesis and trafficking from the endoplasmic reticulum to the plasma membrane, ALK5 and TGFβRII can form non-covalent, non-signaling homodimers [15,16]. The dimeric ligand provides two pairs of distinct binding sites for each pair of receptor, and stabilizes a hetero-tetrameric receptor complex presumably after appropriate rotation of each receptor along the plane of the plasma membrane [17]. The stable hetero-tetrameric complex then signals and is internalized to the endosome. The assembly of the TGF-β receptor differs from the assembly of the BMP receptor complex [17]. The TGF-β ligand binds to TGFβRII and then ALK5 is recruited to the complex in a cooperative manner, by making contacts with both ligand and specific sequences of TGFβRII, which stabilize the complex and are critical for signal transduction. The BMP type II and type I receptors make distinct contacts with the ligand in the absence of receptor–receptor contacts or cooperative assembly, and the localization of the BMP receptors in plasma membrane regions is thought to drive their assembly. Factors that regulate assembly of the hetero-tetrameric receptor complex are of significance for signaling and might contribute to abnormal signaling during disease, such as cancer. Examples include the neurotrophin receptor TrkC and better so its oncogenic form that binds to TGF-β and BMP type II receptors and blocks formation of functional receptor complexes [18,19]. In a distinct manner, the pseudoreceptor BAMBI, which lacks a proper kinase domain, associates with TGF-β, activin and BMP type II receptors and limits proper signaling via normal type I receptors during development [20]. BAMBI's expression is suppressed in the fibrotic liver, which promotes the pro-fibrotic actions of TGF-β signaling [21].

Efficient ligand coordination with the two receptor kinases is often facilitated by accessory receptors that can act in various modes: they can recruit bioactive ligand to the vicinity of the signaling receptor based on their higher affinity for the ligand, they can form complexes with the signaling receptors and ligand, and they can also bind to extracellular antagonists thus facilitating the binding of ligands to the receptor kinases. Such co-receptors include plasma membrane tethered proteoglycans (e.g. the TGF-β type III receptor betaglycan that also binds to inhibin and BMPs) [22,23], glycoproteins (e.g. the TGF-β type III receptor endoglin that modulates TGF-β and BMP signals in endothelial cells) [2], or glycosyl-phospho-inositol-linked receptors (e.g. the Nodal co-receptor Cripto that also binds TGF-β and inhibits its signaling pathway) [24].

When TGF-β ligands bind to the signaling receptor kinases, then the type II receptor, which has a constitutively active kinase activity, trans-phosphorylates the GS (glycine-serine)-rich segment proximal to the transmembrane domain of the type I receptor, at multiple serine residues (Fig. 1) [25]. This trans-phosphorylation reaction changes the conformation of the GS domain, which unfolds and results in the release of negative regulatory chaperones such as FKBP12, thus allowing further conformational change of the type I receptor kinase domain and hence, its catalytic activation. The activated type I receptor then catalyzes phosphorylation of downstream signaling effectors of the Smad family (Fig. 1). In addition, the activated type I receptor catalyzes many other signaling events, as we discuss below. Thus, the type I receptor is an essential molecule in the signaling flaw downstream from the ligand-type II receptor complex. Although the functional attributes of the type II receptors appear somewhat limited, recent examples reveal novel functions for these receptors. For example, TGFβRII directly phosphorylates the polarity protein Par6, which destroys epithelial polarity and induces a migratory cell phenotypes (Fig. 1) [26]. Moreover, TGFβRII can be phosphorylated by the tyrosine kinase Src, which leads to recruitment of the adaptor protein Grb2 to the receptor, and assembly of signaling complexes that regulate receptor function, tumor cell migration and invasiveness [27].

4. Smad molecules form a central intracellular engine

The activated type I receptor directly phosphorylates the C-terminal di-serine motif of members of the Smad family [28]. These receptor-activated Smads (R-Smads) exhibit specificity in their interaction with type I receptors in the family and thus allow a classification of the signaling pathways. Accordingly, the R-Smads Smad2 and Smad3 recognize the L45 loop region of ALK4, ALK5 and ALK7 (type I receptors for TGF-β, activins, and some of the GDFs, e.g. GDF8/myostatin), while the R-Smads Smad1, Smad5 and Smad8 show specificity for the L45 loop of ALK1, ALK2, ALK3 and ALK6 (type I receptors for BMPs, GDFs and AMH) [29–31].

C-terminal phosphorylation of the R-Smads leads to a conformational change of the whole protein and primarily of the C-terminal conserved domain, called Mad-homology 2 (MH2) domain [25]. This conformational change allows the phosphorylated R-Smad to oligomerize with itself or with

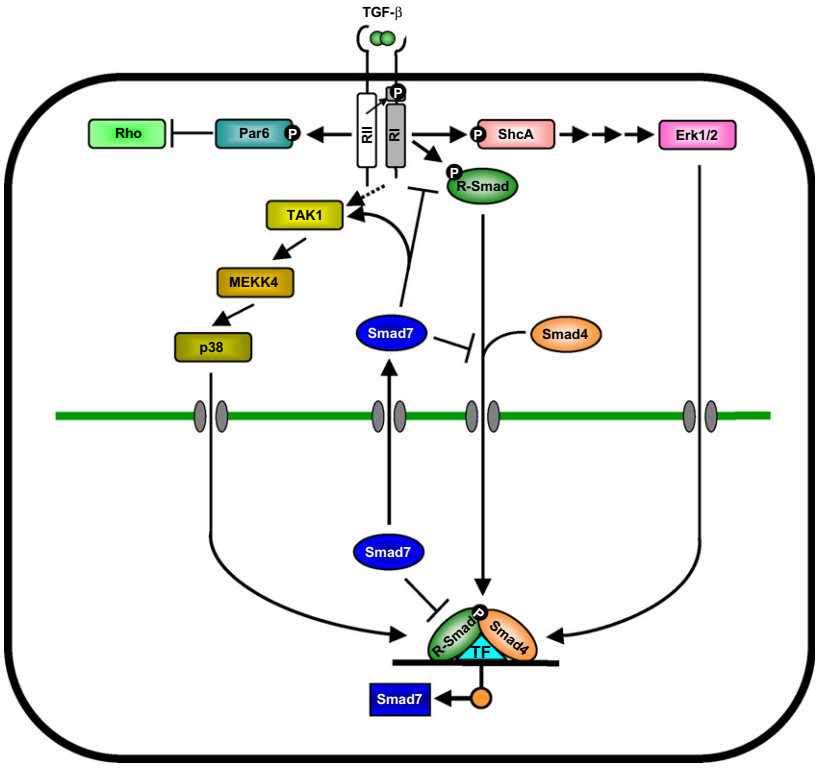

Fig. 1. The TGF-β signaling pathway with Smad and non-Smad branches. Dimeric TGF-β binds to its type II (RII) and type I (RI) receptors, inducing trans-phosphorylation (arrow) of RI by RII. RII phosphorylates the polarity protein Par6, which regulates Rho GTPase degradation and actin dynamics. RI phosphorylates the adaptor ShcA, which leads to the activation of the Ras-MAPK Erk1/2 pathway (multiple arrows) that feeds to the various gene responses of the pathway. RI phosphorylates R-Smads that oligomerize with Smad4 and regulate transcription in the nucleus. One of the target genes of the pathway is the inhibitory Smad7, which blocks TGF-β signaling at multiple levels (R-Smad phosphorylation by RI, Smad oligomerization and degradation, nuclear Smad activity). RI or RII (not yet known-dotted arrow) activate TAK1, which activates MEKK4 and downstream MAPK p38, also providing signals to gene regulation. Smad7 plays a positive role in activation of the TAK1 cascade.

other R-Smads, and, most importantly, with another class of Smad family proteins, the Co-Smad, which is represented by a unique gene product in mammals, the Smad4 (Fig. 1). Smad4 is not phosphorylated by the type I receptors and characteristically lacks the C-terminal di-serine motif [28]. However, Smad4 has a highly conserved C-terminal MH2 domain that interacts with the active conformation of the MH2 domain in the phosphorylated R-Smads, as proven by crystallographic studies of Smad1, Smad2, Smad3 and Smad4 [25]. The hetero-oligomer of R-Smads with the Co-Smad has been crystallized as dimer or trimer. Based on the trimer model, two phosphorylated R-Smads assemble with one Smad4, as Smad4 replaces one of the phosphorylated R-Smads in the R-Smad homo-trimer [32].

The R-Smad/Co-Smad oligomers can assemble either in the cytoplasm or in the nucleus, since all Smad proteins continuously shuttle in and out of the nucleus as we discuss later. Whether there is functional significance ascribed to the location of assembly of the oligomeric Smads is an interesting open question. The Smad oligomer serves as a molecular platform for the organization and execution of many nuclear reactions that instruct the chromatin how to regulate transcription of a multitude of gene targets (Fig. 1), which are different from tissue to tissue and from organism to organism. Context-dependent responses demand an integration of information about the developmental status of the responding cell and this is achieved by various post-translational modifications of the Smad complex in the nucleus. Molecular information is relayed by the Smad complex to chromatin by at least three specific ways: binding to DNA, interaction with other DNA-binding transcription factors, and association with chromatin remodelers and transcriptional co-activators or co-repressors [25].

Smads bind with low affinity to DNA sequences named the Smad-binding elements (SBEs) that include the minimal motif 5′-GTCT-3′ or its complementary 5′-AGAC-3′, to which primarily Smad3 binds, or a loosely defined G/C-rich motif to which Smad4 and BMP-specific R-Smads seem to bind [25]. The DNA-binding domain of Smads resides in their conserved N-terminal MH1 domain and consists of a β-hairpin loop structure. Smad2, one of the R-Smads of the activin/TGF-β/nodal family, is expressed in two alternatively spliced forms, a long form and a shorter form that lacks exon 3 (Δex3 form). Exon3 of Smad2 provides a unique amino acid sequence in very close apposition to the β-hairpin loop, which is thought to perturb the structure of the β-hairpin loop so that full length Smad2 fails to bind to DNA [34,35]. In contrast, the shorter Δex3 form of Smad2 binds the same SBE as that defined for Smad3, with essentially the same affinity. Binding of Smads to DNA can be negatively regulated by phosphorylation of the MH1 domain of Smad2 or Smad3 by kinases such as protein kinase C or calcium-calmodulin kinase II [36,37], and it can be positively regulated by acetylation of the MH1 domain of Smad2 Δex3 or Smad3 by acetyl-transferases such as p300, CBP and P/CAF [38,39]. Whether there is an inverse link between phosphorylation and acetylation of the Smad MH1 domain remains unexplored. In general, regulation of Smad

DNA-binding is poorly understood and deserves further exploration.

The Smad-interacting transcription factors provide the necessary means for specificity or selectivity of the target gene regulatory elements, as the SBEs are highly abundant throughout the genome, yet each of the various TGF-β family signaling pathways regulates roughly 300–500 target genes in a given cell type [40]. Genome-wide information of Smad-chromatin association is still missing despite the availability of chromatin immunoprecipitation-genomic microarray technology (ChIP-on-chip). In association with the many available transcriptomic data sets, genome-wide location analyses will provide a more robust framework for the understanding of how TGF-β pathways relay their signals to the genomes of responding cells. Recent in vitro evidence has also highlighted the importance of properly assembled chromatin for the ability of Smad complexes to mediate transcriptional regulation [41]. This is unlike other transcription factors that can stimulate transcriptional initiation from naked DNA templates in vitro, and is probably consistent with the low affinity binding of Smads to naked DNA. The chromatin-based transcriptional function of Smads is determined by their association with chromatin remodeling proteins such as Brg1, a component of the SWI/SNF complex [41,42], ARC105, a mediator complex [43], and p300, CBP, P/CAF, mSin3 and CtBP, general co-activators and co-repressors that recruit histone acetyl-transferases and deacetylases [44].

Based on the above mechanisms, the Smad pathway regulates a number of transcriptomic programs, like for example the cytostatic (epithelial, endothelial or lymphocyte cell cycle arrest), the apoptotic, the migratory, the transcriptional or the negative feedback programs [40]. This aspect of TGF-β signaling has been thoroughly reviewed over the past few years [14,40,44–46].

5. Feedback by inhibitory Smads

One of the best understood immediate-early gene targets of TGF-β, activin and several BMP pathways is the gene encoding the inhibitory Smad7 (I-Smad) (Fig. 1) [47,48]. Smad7 together with its relative Smad6 constitute the most divergent Smad members, with a poorly conserved N-terminal domain, only distantly related to the MH1 domains of other Smads, a middle linker and a highly conserved C-terminal MH2 domain [28]. I-Smads, like Smad4, lack the C-terminal di-serine motif and are not phosphorylated by the type I receptors.

Smad7 is the best studied among the two I-Smads. Smad7 resides in the nucleus and in addition to its transcriptional induction, TGF-β signaling mobilizes a translocation of Smad7 from the nucleus to the cytoplasm [49]. Smad7 is exported to the cytoplasm together with its companion E3 ubiquitin ligases Smurf1 and Smurf2, and eventually associates with the signaling receptor complex [50–52]. Binding of Smad7 to the type I receptors competitively blocks the recruitment of R-Smads to the same receptors, and thus gradually reduces phosphorylation and activation of the R-Smads (Fig. 1) [53]. Smad7 bound to the type I receptor ALK5 also recruits the phosphatase PP1α which dephosphorylates ALK5, and thus downmodulates its signaling activity [54,55]. Furthermore, Smad7 brings its associated ubiquitin ligases Smurf1 and Smurf2 to the receptor complex [50,51]. This leads to receptor ubiquitination, which promotes receptor internalization through lipid rafts and eventual receptor degradation in lysosomes (Fig. 2) [56].

Smad7 and Smad6 can form complexes with other Smad proteins, especially with Smad4; this leads to dissociation of the R-Smad/Co-Smad complexes (Fig. 1) [57], and also modulates ubiquitination and degradation of R-Smads and Co-Smads found in these common complexes [58]. Finally, Smad7 inhibits TGF-β signaling in the nucleus, by binding to Smad-transcription factor complexes on chromatin, and blocking nuclear transmission of the TGF-β signal (Fig. 1) [59]. Thus, I-Smads represent multifaceted adaptor proteins that organize several negative regulatory reactions that ensure downregulation or shut down of TGF-β signaling.

Despite this prominent role of I-Smads as negative regulators, recent evidence illustrates the importance of Smad7 as an organizer of additional signaling proteins around the type I receptor, that lead to regulation of MAPKs and Rho GTPases, during apoptotic and cytoskeletal responses to TGF-β (Fig. 1) [60,61]. The mechanism that causes the switch of Smad7 from a negative to a positive regulator of TGF-β signaling might well rely on differences in the timing of events, post-translational modifications of Smad7, and recruitment of yet uncharacterized regulatory proteins to the type I receptor, an area worth investigating deeper.

6. TGF-β receptors activate additional, non-Smad signaling effectors

While signaling downstream of TGF-β family receptors firmly involves the Smads, growing evidence places additional signaling effectors operating under the control of these receptors [62]. Recently, clear links between the activity of the type I and type II receptors via phosphorylation or direct docking of such effector proteins have been made. Since this topic has also been abundantly reviewed [62,63], we highlight here only few examples that illustrate distinct mechanistic paradigms of signaling by the TGF-β family receptors to proteins different than the Smads.

Possibly the two most direct links between TGF-β receptors and alternative signal transducers involve the phosphorylation of new substrates by the receptors. As summarized above, an alternative substrate of the TGFβRII is the polarity complex protein Par6 [26]. ALK5 resides in tight junctions of polarized epithelial cells and makes complexes with integral components of the tight junction such as occludin [26]. TGFβRII resides in association with E-cadherin in adherens junctions [64]. When the signaling receptor complex forms, TGFβRII phosphorylates Par6, which leads to recruitment of the ubiquitin ligase Smurf1 (also described under the negative control of TGF-β receptors by Smad7); Smurf1 now ubiquitinates a novel substrate, the small GTPase RhoA, which regulates the dynamics of tight junction assembly and also the cytoskeleton that directly supports the assembly and function of such junctions (Fig. 1) [26]. Proteasomal degradation of RhoA mediates tight junction dissolution and architectural remodeling of the epithelial cell as we discuss later.

A second prominent example is based on the intrinsic and weak tyrosine phosphorylation activity of ALK5 and TGFβRII. The functional relevance of tyrosine auto-phosphorylation by TGFβRII remains elusive [65]. In contrast, ALK5 phosphorylates the adaptor protein ShcA on tyrosine

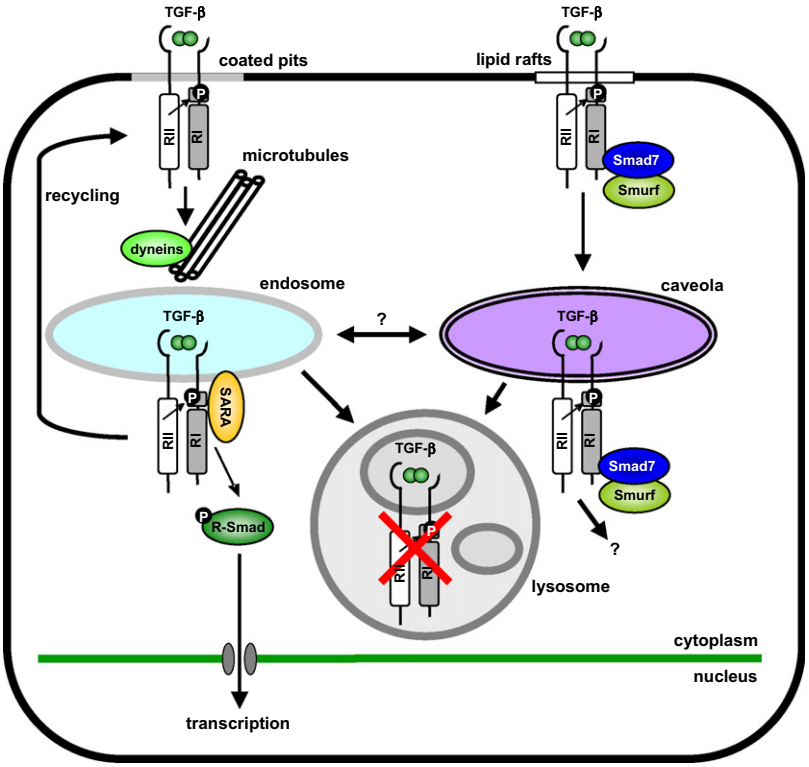

Fig. 2. TGF-β receptor trafficking. TGF-β receptor complexes residing in membrane areas that form coated pits (grey line) are internalized possibly via action of the motor proteins dyneins along microtubules and localize at endosomes. R-Smad phosphorylation is promoted in endosomes based on the action of the endocytic protein SARA, leading to transcriptional signaling. Endosomal receptors may recycle back to the plasma membrane or progress towards lysosomes where the ligand–receptor complex is degraded (red cross). TGF-β receptor complexes residing in membrane areas called lipid rafts (white line) associate with Smad7 and Smurf ubiquitin ligases and are internalized to caveolae. These receptor complexes progress to lysosomes for degradation and whether they are involved in signal transduction remains unknown (question mark). The possibility for intercommunication between the endosomal receptor pool and the caveolar receptor pool is not established and is marked with a double arrow with question mark.

and serine residues (Fig. 1) [66]. The tyrosine-phosphorylated ShcA then recruits Grb2 via its Src homology 2 domain, which then mobilizes the well known MAPK signaling cascade. The biological context under which TGF-β might choose to signal via this ShcA-Grb2-MAPK pathway in parallel to the activation of the Smad pathway requires deeper understanding.

A third example is the activation of the TGF-β-activated kinase 1 (TAK1) by TGF-β and BMP receptors (Fig. 1). TAK1 is a MAPK kinase kinase and despite its original discovery as signaling effector of TGF-β receptors [67], it has been firmly linked to the signaling of pro-inflammatory interleukins and Toll-like receptors [68]. TAK1 represents a special type of kinase whose activity is regulated by ubiquitination during regulation of the pro-inflammatory NF-κB pathway. During TGF-β signaling, TAK1 activates the downstream MAPK p38 and plays important roles in the induction of apoptosis in tumor and normal cells [60,69]. Interestingly, one link between TAK1 and the TGF-β receptors has been shown to be the adaptor protein Smad7, which directly binds to ALK5 and to TAK1 [60]. TAK1 also signals downstream of BMP receptors and the E3 ubiquitin ligase and inhibitor of apoptosis XIAP has also been linked in this pathway [70]. TAK1 signaling downstream of BMP receptors plays roles during early *Xenopus* development and a mechanism leading to specific activation of the TAK1-p38 MAPK signaling cascade is the oligomerization of BMP receptors in response to ligands [71]. Of higher importance though will be the elucidation of a molecular mechanism by which the TGF-β or BMP receptors might regulate the catalytic activity of TAK1 so that its downstream signaling cascade is mobilized. Such a mechanism might involve direct phosphorylation by the receptors, or alternatively, it might involve signaling complex assembly based on adaptor proteins like Smad7. The recent evidence that TAK1 directly interacts with TGFβRII and indirectly with ALK5 does not yet solve this exciting open problem [72].

7. Coordinated signaling by Smad and non-Smad pathways

A frequent question in the understanding of TGF-β pathways is the relative contribution of Smads and alternative effectors during a particular biological process. One possibility is that Smad signaling primarily affects transcriptional regulation, whereas non-Smad signaling might modulate cytoplasmic processes. The true picture points to integration of several pathways. For example, TGF-β or BMP signaling via TAK1 and p38 MAPK contributes to specific gene regulation in parallel to Smad signaling [73]. On the other hand, TAK1 was found to interact with all Smad proteins and thus, to negatively regulate Smad signaling and its effects on osteogenesis [74]. TAK1 also phosphorylates and regulates the stability of the Smad-interacting protein SnoN [75], which acts as an oncogene or tumor suppressor by regulating various facets of Smad signaling in the cytoplasm and the nucleus [76]. TGF-β, via

Smads and various ubiquitin ligases leads to transient degradation of SnoN, which allows robust nuclear Smad signaling, as SnoN generally represses the transcriptional activity of Smads. However, TAK1 also enhances SnoN degradation [75], and it is worth examining whether phosphorylation of SnoN by TAK1 might be a priming event required for the Smad-dependent recruitment of ubiquitin ligases to SnoN.

Thus, Smad and non-Smad signaling seem to coordinate common target events. A good example illustrating this model is based on the structurally flexible linker domain of Smad proteins, that links the N-terminal MH1 with the C-terminal MH2 domains [28]. The linker domain is proline- and serine/threonine-rich. MAPKs such as Erk1 and Erk2 [77,78], cyclin-dependent kinases [79], and synthase glycogen kinase 3β (GSK3β) [80,81], phosphorylate distinct and often multiple amino acid residues within the linker domain of R-Smads such as Smad1, Smad2 or Smad3. Linker phosphorylation seems to be a priming event for the recruitment of the ubiquitin ligase Smurf1 to specific proline-tyrosine motifs of the R-Smad linker domain [80,84]. The Smurfs eventually ubiquitinate and degrade the R-Smads, thus leading to a shut off mechanism of Smad signaling. This mechanism can be counteracted by phosphatases such as the small C-terminal domain phosphatases (SCPs) that dephosphorylate the R-Smad linker and enhance Smad signaling [82,83]. In Smad1, GSK3β phosphorylates the linker, which is required for the subsequent phosphorylation of the linker by Erk1/2, which leads to Smad1 ubiquitination and proteolytic degradation in proteasomal factories associated with the centrosome [80]. The stability of Smad3 is also regulated by GSK3β, which phosphorylates the MH1 and linker domains, but phosphorylation of the MH1 domain rather than the linker seems to correlate better with Smad3 degradation [81]. Such mechanisms can partially explain an antagonism of TGF-β signaling by mitogenic factor signaling that activates Erk MAPK [77,78]. Accordingly, epidermal growth factor, hepatocyte growth factor or fibroblast growth factor (FGF) or alternatively, oncogenic forms of Ras, a downstream signaling protein of these mitogens that signal via receptor tyrosine kinases (RTKs) and activate Erk1/Erk2, lead to R-Smad linker phosphorylation and degradation, thus negatively regulating the TGF-β pathway.

On the other hand, during development, TGF-β and FGF signaling cooperate and FGF activates the CK1ε/δ kinases that phosphorylate the tumor suppressor transcription factor p53 [85]. This phosphorylation enables p53 to associate with Smads and regulate critical target genes that specify the mesodermal germ layer in *Xenopus* embryos or cell cycle inhibitor genes like *p21*, that mediate epithelial growth arrest by TGF-β. Thus, signaling pathways such as those initiated by RTKs can either negatively regulate the Smads or positively activate Smad co-factors such as p53. The time or context-dependence of such cross-talk that defines binary choices of the TGF-β signaling pathways requires critical future attention.

On a different scenario, Smad3 directly interacts with other signaling proteins, such as protein kinase A (PKA) or protein kinase B (PKB/Akt), whose catalytic activities are directly regulated by the association with Smad3 [86–89]. Both of these kinases participate in highly complex signaling cascades initiated by alternative extracellular factors such as hormones and neurotransmitters that signal via G-protein coupled receptors or RTKs that in addition to MAPK cascades activate the phospho-inositide 3′-kinase (PI3K)-Akt pathway.

These examples illustrate the highly integrated nature of signaling cascades initiated by TGF-β family receptors. These cascades provide cross-talking points with other signaling pathways, and it seems that each one of these pathways cross-checks the progression of signal transduction of the other pathway. Positive and negative signaling inputs provide the means for highly controlled and progressive signaling.

8. TGF-β receptor trafficking

The TGF-β receptor complex is thought to reside in either plasma membrane areas that assemble coated pits or in the so called lipid rafts (Fig. 2) [56]. Single molecule measurements of TGFβRII and ALK5 lateral diffusion concluded that heteromeric receptor assembly preferentially occurs at lipid rafts [90]. In polarized epithelial cells, the receptors can be associated with adherens and tight junctions as described above. In fact adherens and tight junctions represent distinct microdomains of the plasma membrane that have major characteristics of lipid rafts [91]. The dynamic distribution of the receptors within such membrane microdomains could possibly be driven by underlying cytoskeletal elements; however, this topic remains largely underexplored. Only few reports so far shed some light to this question. A dynein protein light chain subunit, the protein km23-1, associates with signaling TGF-β type I receptors and localizes in early endosomes [92]. km23-1 is functionally required for physiological TGF-β signaling as examined by various biological assays. A different dynein light chain subunit, the protein Tctex2β, interacts with the accessory receptor endoglin and TGFβRII and mediates their microtubule-based transport [93]. Proper cellular levels of Tctex2β regulate TGF-β/endoglin signaling in endothelial cells.

According to one prominent model, TGF-β receptor complexes associated with coated pits endocytose towards early endosomes [56,94], where R-Smads (of activin/TGF-β/nodal pathways) are presented by endosomal proteins such as SARA, resulting in R-Smad phosphorylation and activation (Fig. 2) [95]. During BMP signaling, receptor internalization by clathrin-mediated endocytosis is followed by R-Smad presentation to the receptors by the SARA-related protein endofin [95]. On the other hand, BMP receptor signaling towards the TAK1-p38 pathway initiates from receptor complexes localizing in lipid rafts [96]. Differences between TGF-β and BMP receptor localization on the plasma membrane may well be associated with the differences in ligand–receptor assembly as explained earlier. Our increasing understanding of the role of TGF-β receptor endocytosis in promoting this signaling pathway is also nicely illustrated by a recent report that identified the Rab5 guanine exchange factor (GEF) called RIN1, as a strong promoter of TGF-β signaling by enhancing receptor endocytosis [97]. Furthermore, TGF-β signaling induces the transcriptional repressor Snail, which directly represses expression of the *RIN1* gene, thus providing another negative feedback mechanism for the time-dependent control of this pathway. It should be emphasized that all trafficking pathways are highly dynamic, and thus, defining with accuracy whether Smads get phosphorylated only on the plasma membrane, en route to early endosomes or only in endosomes has been technically demanding and still not properly resolved.

According to the same model of TGF-β receptor internalization, when ligand-bound receptors associate with lipid rafts,

they recruit Smad7–Smurf complexes, leading to receptor internalization via caveolae and degradation in lysosomes (Fig. 2) [56]. However, as the various cytoplasmic trafficking vesicles seem to communicate with each other, it will be interesting to examine whether certain pools of receptors traffic between the endosomes and caveolae or vice versa, thus providing feedback about biochemical events in each locale (Fig. 2). The multifunctional role of Smad7 provides sufficient reasons that would support such a model. The endosomal route can also lead to the lysosome or alternatively generate vesicles that either recycle to the plasma membrane or that could even be fused to the endoplasmic reticulum-nuclear envelope compartments. These are attractive possibilities not yet investigated, whereby signaling TGF-β receptors could give feedback to newly synthesized pools of TGF-β receptors, or signal from the nuclear envelope.

9. Nucleocytoplasmic shuttling of Smads and its control by the cytoskeleton

The trafficking of components in the TGF-β signaling network is even more perplexed when it comes to the Smads. As we already described, Smad proteins shuttle between the cytoplasm and the nucleus and current evidence suggests that at least engineered GFP-tagged Smads constantly shuttle with different kinetics for each specific Smad [98]. Using a protein complementation assay of Smad proteins fused to fragments of fluorescent proteins in developing *Xenopus* embryos, Smad2 homomers and Smad2–Smad4 heteromers were detected in nuclei of cells that were responding to activin signaling, whereas Smad4 homomers remained cytoplasmic [33]. Furthermore, functional Smad2–Smad4 heteromers accumulated in the nuclei of developing embryonic cells only after a certain developmental stage, which was independent from the time the embryos were subjected to activin treatment, suggesting a developmental context-dependent control of the nuclear accumulation of the heteromeric complex. Analysis of Smad trafficking in the cytoplasm showed that Smad2 associates with kinesin-1 and requires microtubules for movement towards signaling receptors (Fig. 3) [99]. In fact, all Smads are known to associate with microtubules and the functional significance of this remains poorly analyzed [100]. Similar to ALK5, the dynein light chain km23-1 also associates with Smad2 and is required for proper nuclear accumulation of Smad2 [92]. Thus, a new scenario can be considered whereby nuclear Smads traffic towards signaling receptor complexes on early endosomes using microtubules and kinesin motors, whereas the inverse

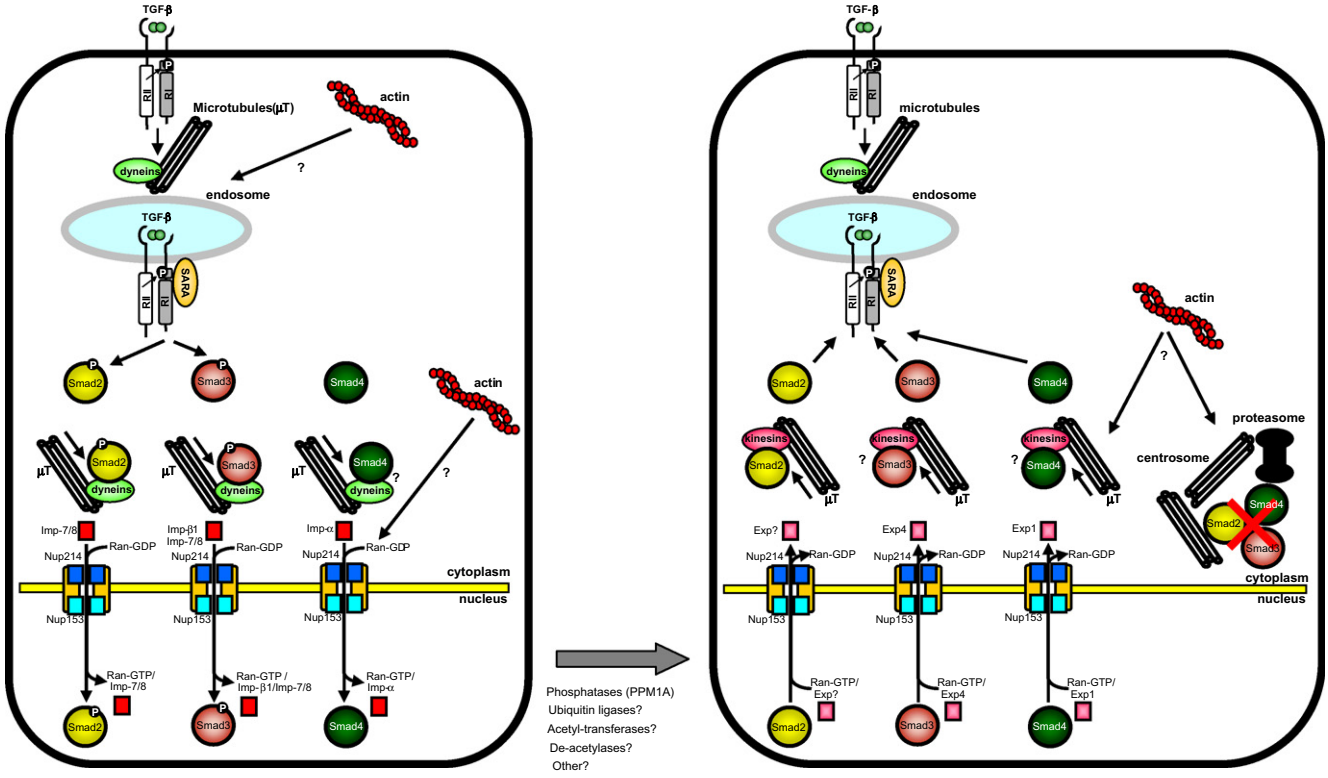

Fig. 3. Smad trafficking. Left panel: TGF-β receptors are internalized to endosomes via microtubules and dyneins. A role for actin microfilaments has been suggested but not yet firmly established (question mark). Smad proteins move towards the nuclear pore via microtubules and dyneins, although this scenario is not established for Smad4, neither a role for actin microfilaments in this process (question marks). The Smads interact with importins (Imp) and nucleoporins (Nup) and translocate to the nucleus by Ran-dependent catalysis and release of the importins in the nucleus. Various nuclear events can trigger exit from the nucleus to the cytoplasm (middle grey arrow). Among those listed only the action of phosphatases has been firmly linked to the nuclear export of Smads. Right panel: Nuclear, de-phosphorylated Smads utilize exportins (exp, not yet identified for Smad2-question mark), the catalytic activity of Ran and interaction with nucleoporins (Nup) to exit to the cytoplasm. Via microtubules and kinesins (evidence exists only for Smad2, question marks for Smad3 and Smad4), Smads reach the signaling receptors and get re-activated for a new round of signaling. Alternatively, the exported Smads are recruited to the centrosome and are degraded by proteasomes (red cross). A role for actin microfilaments in these processes is not known (question mark). The figure does not differentiate between shuttling of Smads in the absence and presence of ligand stimulation as discussed in the text.

trafficking towards the nucleus also depends on microtubules but involves dynein motors (Fig. 3). On the other hand, microtubules have also been proposed to trap Smads in the cytoplasm, while their release can be regulated by proteins such as connexin43, which competes for microtubule binding with the Smads [101]. The possibility that such a mechanism provides a switch that regulates the mobility of Smads along microtubules remains to be clarified.

Microtubule assembly starts from organizing centers, such as the centrosome. In fact, Smads have been shown to undergo proteasomal degradation in centrosomes (Fig. 3) [80], associate with the spindle in mitotic cells and also decorate chromosomes during metaphase. Interestingly, the protein complementation study in *Xenopus* embryos (summarized above), revealed Smad2–Smad4 heteromeric complexes in association with chromosomes, every time the embryonic cells divided, and independent of whether these cells were responding to a TGF-β family ligand [33]. This may reflect an effort to properly segregate Smads to daughter cells after mitosis or might suggest that Smads regulate the process of mitosis and cytokinesis per se. *Drosophila* embryos provide a positive lead in this direction, as epithelial cells of the wing imaginal discs contain endosomes enriched in the protein SARA, the ligand Dpp and its type I receptor Thickveins. During each cell division of the growing imaginal disc, the ligand–receptor–SARA complex together with endosomes segregate to daughter cells via microtubules of the mitotic spindle [102]. This mechanism provides a fresh clue as to how developing tissues can spread their TGF-β signaling machineries across the organ and complements the processes of transcytosis and cell surface-mediated transport of ligands. Thus, studying the subcellular distribution of Smads during the cell cycle of various cell types is an appealing topic for present investigation.

Trafficking of Smads together with endocytic vesicles as multimolecular protein complexes along microtubules, does not capture the whole picture of Smad shuttling. Conserved, lysine-rich nuclear localization signals (NLS) have been identified in all Smads [28]. The NLS resides in the MH1 domain very close to the DNA-binding β-hairpin loop and is recognized by transporters, importin-β for Smad3 and importin-α for Smad4 [103–106]. Transportin-dependent import of Smad3 has been proven by in vitro import assays in partially permeabilized cells with intact nuclei, while the biological relevance of the NLS has been defined by mutational experiments that established the necessity of the lysine-rich motifs for Smad nuclear accumulation. Furthermore, the functional role of the small GTPase Ran was firmly established by these in vitro assays [103]. Ran, in its GTP-bound form, facilitates the dissociation of Smad3 from importin-β in the nucleus and assists the recycling of importin-β back to the cytoplasm (Fig. 3). Using in vitro import assays, the necessity of specific transporters was refuted, while Smad2, Smad3 and Smad4 were shown to bind to the phenylalanine/glycine (F/G)-rich repeats of specific nucleoporins of the nuclear pore (Fig. 3) [107,108]. Smad2 and Smad3 contact nucleoporins via their MH2 domain hydrophobic surface, while Smad4 interacts with nucleoporins via a not well defined epitope on the surface of the whole protein. In vitro import experiments of specific Smad domains mapped the functional import activity of Smads (or in other words, a domain that could carry NLS-like activity) to the MH2 domain instead of the MH1 domain. The direct physical interaction between the nucleoporin Nup214 and Nup153 F/G-repeat fragments was demonstrated only for the MH2 domain of Smad2, but not for the corresponding domains of Smad3 or Smad4 [107,108]. Interestingly, the transportins like importin-β, transport their cargo through the nuclear pore by making multiple direct contacts with the F/G-repeats of the nucleoporins. Thus, it is possible that Smads are transported to the nucleus via specific transportins while also making direct contacts with specific nucleoporins of the nuclear pore.

Smad2 is an interesting Smad, as its unique exon3 that perturbs binding to DNA, also perturbs its recognition by importin-β [103]. Thus, Smad2 would not be able to make contacts with importin-β via its MH1 domain, while it could contact the nucleoporins via the hydrophobic surface in its MH2 domain. Unfortunately, the NLS sequence of Smad2 has not been mutated to examine the functional relevance of this motif in the nuclear import of this protein in intact cells. Interestingly, a recent RNAi-based screen for novel regulators of the nuclear import of Mad, the *Drosophila* R-Smad of the Dpp pathway, identified a novel transportin, moleskin, the *Drosophila* homologue of two human transportins, importin-7 and importin-8 (Fig. 3) [109]. RNAi experiments in the fly clearly established the role of endogenous moleskin on nuclear import of phosphorylated Mad and also the role of importin-7 and importin-8 on the nuclear import of Smad1, Smad2 and Smad3 in human cancer cells in response to BMP or TGF-β signaling, respectively. This latter study also excluded the role of the *Drosophila* homologue of importin-β, ketel, in the nuclear import of phosphorylated Mad in fly cells, but unfortunately did not test for the role of importin-β on import of Smad1 or Smad3 in mammalian cells. These latest in vivo data support an interesting model whereby constitutive Smad import does not require transportins, and presumably is based on direct contacts with nucleoporins, while nuclear import of phosphorylated R-Smads, requires the transportins.

This conclusion brings us to an important current model according to which, Smads continuously shuttle in and out of the nucleus, and at any given moment, most of the Smad molecules distribute in the cytoplasm [13]. This model is based on studies of Smad2 and Smad4 and remains to be applied to Smad3 or to BMP-specific Smads. In addition, all kinetic analyses rely on transfected GFP-tagged Smads that seem to approximate rather well the behavior of endogenous Smads, however, they represent a condition where the specific Smad concentration has at least been doubled within the transfected cells [98]. This kinetic analysis demonstrated that nuclear export is a more critical determinant during Smad shuttling. In other words, Smads enter the nucleus almost spontaneously (presumably by utilizing their affinity to nucleoporins), while their export is a slower process. Thus, upon activation of TGF-β pathways by ligands, activated R-Smad/Co-Smad complexes accumulate in the nucleus because their nuclear export is blocked (presumably by masking of specific nuclear export signals within the complex). The residence of the nuclear Smad complexes then can be regulated by nuclear phosphatases such as PPM1A (for Smad1, Smad2 and Smad3) [110,111], SCPs (for Smad1) [112] and pyruvate dehydrogenase phosphatase [113] that dephosphorylate the C-terminal di-serine motifs of R-Smads, leading to monomeric Smads that follow their constitutive shuttling cycle. Based on experiments with inhibitors of the activin/TGF-β/nodal type I receptors, the nuclear residence of Smad complexes seems to depend on the time that the TGF-β receptors remain active, since inhibition of the type

I receptors led to rapid redistribution of the nuclear Smads throughout the cell [114]. This is an attractive model that directly links the activity of the receptor complex to the relative concentration of Smad complexes in the nucleus. It relies primarily on cycles of phosphorylation and dephosphorylation of the C-terminal di-serine motif, but it is likely that other post-translational modifications of Smads also have important roles, such as phosphorylation of the linker and other domains, ubiquitination and local degradation, acetylation and de-acetylation, or yet uncharacterized post-translational modifications.

Despite its current incompleteness, the shuttling model emphasizes the role of nuclear export of Smads. Indeed, nuclear export signals (NES) were first defined in Smad4, which carries a leucine-rich NES in its linker domain [115,116]. This NES is recognized by exportin-1/CRM1 (Fig. 3), and when mutated or when cells are incubated with the CRM1-specific inhibitor leptomycin B, then Smad4 distributes exclusively in the nucleus of cells that were either stimulated or not with ligands of the TGF-β family. One fact that contradicts the importance of the Smad4 NES is based on the kinetic analysis previously described, which suggests a steady-state Smad4 distribution that is both cytoplasmic and nuclear, and proposes that the functionality of the Smad4 NES is regulated by unknown mechanisms. Recently, the biological significance of Smad4 export and of the whole Smad shuttling model has been challenged, since mice with a knock-in mutation in the Smad4 NES, which led to stronger nuclear residence of Smad4, did not exhibit any obvious phenotype [117], suggesting that nuclear export of Smad4 via this NES might not be of importance for embryonic development and adult tissue homeostasis. In analogy with Smad4, a bipartite, leucine-rich NES found in the MH2 domain, regulates the export of Smad1 [118]. And finally, a non-classical NES sequence residing also in the MH2 domain of Smad3 was shown to mediate its nuclear export via the novel exportin-4 and with the catalytic power of the Ran GTPase (Fig. 3) [119].

A related topic to the mechanisms of regulation of Smad shuttling is the importance of tethering factors that might trap Smads in the cytoplasm or the nucleus. Such roles have been proposed for the endocytic protein SARA in the cytoplasm [120], and for yet unidentified chromatin-associated factors in the nucleus [98]. However, all current evidence disfavors the existence of factors that completely trap Smad proteins in a specific compartment. It is more likely that any protein that associates with the Smads either in the cytoplasm or in the nucleus might regulate the timing or residence of the Smad in a specific cell compartment. Such regulatory proteins can be TRAP-1, a Smad4 chaperone that controls the recruitment of Smad4 to TGF-β receptors, TLP1 and Erbin, two Smad2 and Smad3-interacting regulators, which limit the incorporation of Smad2 or Smad3 into signaling complexes with the Co-Smad, and ELAC2, a Smad2 nuclear partner that facilitates transcriptional signaling [121–124].

Attractive systems that could regulate the mobility and function of Smads in the cell are cytoskeletal filaments and nucleoplasmic microdomains. However, with the exception of the role of microtubules that seem to provide routes for the rapid movement of Smads and/or vesicles carrying TGF-β receptors in the cytoplasm, no other role of the actin microfilament system, the intermediate filament system of keratins or vimentin or of any specific regulator of nuclear architecture has yet been reported. Thus, additional work on the topic of Smad trafficking is needed before this aspect of regulation of TGF-β signaling is fully understood.

10. TGF-β controls the cytoskeletal machinery to induce cell motility or changes in cell architecture

Although the role of cytoskeletal elements in modulating TGF-β signaling has just started to emerge and relates primarily to microtubules, TGF-β members have a strong and complex impact on the organization of cytoskeletal architecture. This primarily involves the actin cytoskeleton and secondarily certain systems of intermediate filaments [1]. By targeting the actin cytoskeleton, TGF-β signaling seems to aim at least at two different physiological outcomes that are interlinked: first, it changes the overall cellular architecture which has an impact on differentiation and proliferation, and second, facilitates cell motility, prerequisites of which is the altered architectural arrangement and the remodeling of the extracellular matrix to which the cell adheres and migrates on. The change in cellular plasticity is most clearly seen in epithelial cells that undergo epithelial–mesenchymal transition (EMT), a change in their differentiation that promotes cell migration and is biologically linked to embryonic tissue movements and to tumor cell invasiveness and metastasis [125]. During EMT, epithelial cell–cell junctions are dissolved and the underlying actin cytoskeleton is reorganized with prominent formation of focal adhesions and stress fibers interconnecting these focal adhesions so that ''ameboid'' movement of the mesenchymal cells is obtained. During EMT, the intermediate filament system of cytokeratins exchanges to new cytokeratins and to a vimentin-based skeleton. At least in the case of vimentin, we understand that Smad signaling together with cooperating transcription factors induces expression of its gene during mesenchymal differentiation (Fig. 4) [126]. These changes are functionally associated with induced cell mobility towards either a chemotactic gradient of TGF-β ligands or towards a gradient of other chemoattractants like platelet-derived growth factor or chemokines, whose secretion is potently induced by the primary TGF-β stimulus.

We already described the most prominent example of direct signaling by the TGF-β receptors towards the small GTPase Rho, a prominent regulator of actin cytoskeleton dynamics. This mechanism entails phosphorylation of Par6 and ubiquitination by Smurf1 (Fig. 4) [26]. According to the mechanism, TGF-β receptor signaling initiating at tight junctions leads to local disassembly of actin microfilaments intimately associated with the assembly of the junctions, which is required for their effective dissolution. However, direct demonstration of such modulation of actin dynamics that support tight junction assembly in response to TGF-β has not yet been reported. This example illustrates an exciting area for future research. Instead of measuring global effects of TGF-β members on the actin cytoskeleton, which has by now been reported repeatedly, analysis of dynamic changes in subcellular compartments promises the discovery of new mechanisms of regulation of cellular architecture.

Not only RhoA protein levels can be locally degraded in response to TGF-β, but more frequently signals by either TGF-β or BMPs activate small GTPases of the Rho family (e.g. RhoA or Cdcd42) and positively affect the assembly of new actin

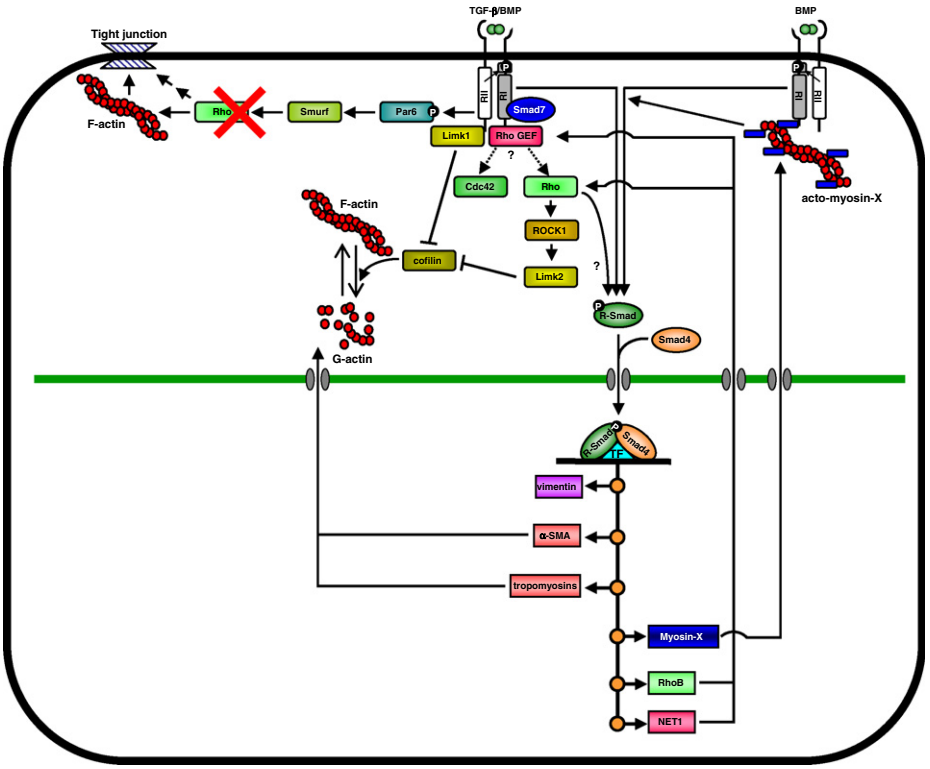

Fig. 4. TGF-β/BMP signaling to the cytoskeleton. The TGF-β receptor, RII phosphorylates Par6, which recruits the ubiquitin ligase Smurf that leads to ubiquitin-dependent degradation of RhoA, a positive regulator of filamentous actin (F-actin) and tight junction assembly. BMP RII binds and activates Limk1 that phosphorylates and inhibits cofilin, an actin depolymerising factor, favoring globular actin (G-actin) accumulation. BMP or TGF-β type I receptors activate small GTPases such as Cdc42 and RhoA or RhoB (Rho) by as yet unknown mechanisms (dotted arrow and question mark), which clearly involve Smad7 and possibly receptor-bound Rho GEFs. Rho activates ROCK1 kinase, which activates Limk2, which acts in a similar fashion as Limk1 in regulating actin dynamics via cofilin. Rho GTPases also play positive roles in promoting Smad signaling, which regulates expression of various genes that regulate the cytoskeleton. Synthesis of vimentin leads to a new intermediate filament cytoskeleton (not shown). Synthesis of α-smooth muscle actin (α-SMA) and tropomyosins contributes to the assembly of new acto-myosin networks that promote cell motility. Synthesis of myosin X contributes to new acto-myosin networks in fillopodia of migrating cells that also recruit the BMP receptor complex and positively regulate BMP-specific Smad signaling. Synthesis of RhoB and NET1 (a Rho GEF) lead to sustained activation of the receptor-Rho pathway.

microfilaments in diverse cell models. Accordingly, the long cytoplasmic tail of the BMP type II receptor BMPRII directly associates with the kinase Limk1 during morphogenesis of dendrites by neuronal cells, and the process gets misregulated in certain cases of pulmonary hypertension [127,128]. Binding of Limk1 to BMPRII leads to activation of its kinase activity, which then phosphorylates cofilin, a factor that depolymerizes filamentous actin (Fig. 4). Phosphorylated cofilin is inhibited and thus actin dynamics shift towards polymerization. In addition to the direct activation of Limk1 by the BMPRII, BMP signaling also activates the Cdc42 GTPase, which also contributes to positive actin microfilament assembly [128].

During embryonic nerve growth cone path-finding, BMPs again affect Limk1 activity and cofilin phosphorylation as cones are attracted towards the BMP source; however, when growth cones reach higher concentrations of the BMP gradient, their receptors reach saturation and the cones are repelled from the BMP source, at which point the cofilin phosphatase slingshot becomes activated, and returns the equilibrium of this pathway back to its starting stage [129]. This elegant mechanism is further regulated by the transient induction of a calcium channel receptor, TRPC1, which leads to calcium-dependent regulation of the calcineurin phosphatase that dephosphorylates and activates slingshot. Similar to BMPRII, ALK5 induces RhoA or RhoB GTPase activity, leading to downstream activation of the kinase ROCK1, which then phosphorylates Limk2 in order for cofilin to be inhibited as described above, and positively affect actin polymerization in fibroblasts and epithelial cells (Fig. 4) [130]. Furthermore, in breast cancer cells that exhibit enhanced motility and metastasis in response to TGF-β, PI3K, the MAPKs Erk1/2 and the small GTPase Rac1 promote actin organization and cell migration, while in transformed fibroblasts TGF-β induces expression of RhoB in order to reorganize actin microfilaments (Fig. 4) [131–133]. A similar interplay of MAPK and Rho GTPase signaling has been established during physiological migration of eyelid epithelial cells and keratinocytes in mice, as demonstrated by knockout of the MAPK kinase MEKK1, which activates downstream Jun-N-terminal kinase and p38 MAPKs to elicit such migration induced by TGF-β or activin [134,135].

The best example of a Smad protein linked to the regulation of Rho family GTPases and actin dynamics comes from prostate cancer cells, where the inhibitory Smad7 acts as an adaptor recruited to ALK5 in order for Cdc42 activity to be enhanced (Fig. 4) [61]. In the same model, TGF-β also induces RhoA and downstream p38 MAPK activation leading to actin polymerization [136]. This mechanism, together with the above examples of Rho GTPase and Limk activation by TGF-β, raise the possibility that TGF-β receptors may serve as organizing

platforms for many signaling proteins that regulate actin dynamics. However, a direct interaction of Rho GTPases or the ROCK1 kinase with TGF-β receptors has not yet been demonstrated. Alternatively, a Rho family GEF might directly associate with TGF-β receptors, becoming activated possibly via phosphorylation by the receptor and then activating a Rho GTPase. A link with Rho family GEFs has already been made, since the Smad3/Smad4 complex in cooperation with Rho and p38 MAPK signaling downstream of TGF-β receptors, on the one hand induces expression of the GEF NET1 (Fig. 4), which accumulates and promotes actin polymerization [137], and on the other hand induces expression of tropomyosins that regulate assembly of a contractile apparatus that serves the motility of the responding cell during the EMT process (Fig. 4) [138]. As TGF-β establishes the mesenchymal phenotype via EMT, it also triggers the nuclear translocation of myocardin-related transcription factors (MRTFs), which interact with Smads and regulate major transcriptional mediators of EMT, such as the Slug repressor [139]. In addition, MRTFs cooperate with serum response factor (SRF) in mediating transcriptional induction of various components of the actin cytoskeleton, including tropomyosin I, caldesmon and α-smooth muscle actin (αSMA).

In a parallel scenario, during EMT at the early stage of embryonic gastrulation, activin/nodal-specific Smad signaling induces expression of two cooperating proteins, the fibronectin leucine-rich repeat transmembrane 3 (FLRT3) and the small GTPase Rnd1, which interact with each other and regulate endocytosis of cadherin from adherens junctions [140]. This process depletes cadherin, a hallmark of EMT, and facilitates cell migration after actin reorganization. While EMT is a process ascribed to epithelial cells, a variety of other cell types, including endothelial cells and fibroblasts can also undergo a similar process that leads to their differentiation into myofibroblasts. Smad signaling in response to TGF-β induces expression of the *SM22α* gene in association with the transcription factor myocardin, or induces expression of the *αSMA* gene in association with transcription factors YB-1 and Sp1 (Fig. 4) [141,142]. SM22α and αSMA are the most characteristic proteins of smooth muscle cells as they build a new actin cytoskeleton with more contractile properties that serves the function of contracting myofibroblasts. Finally, formation of specialized actin structures, called podosomes, underneath the plasma membrane of endothelial cells involves de novo synthesis of cytoskeletal proteins and requires coordinated signaling by the cytoplasmic tyrosine kinase Src, PI3K, Cdc42 and Smads [143]. Such podosomes become enriched in metalloproteases necessary for the remodeling of blood vessels.

Interestingly, two recent reports provide evidence that the cytoskeleton, or some of its key regulators, can regulate TGF-β pathways. In differentiating myofibroblasts, TGF-β activates RhoA, which regulates Smad nuclear accumulation and transcriptional output in parallel to actin remodeling (Fig. 4) [144]. On the other hand, BMPs induce myosin-X transcription in endothelial cells and cause their migration during vessel formation [145]. Interestingly, newly synthesized myosin-X co-localizes with the BMP type I receptor ALK6 into filopodia, and the receptor-myosin-X complex becomes motile (Fig. 4). This process is required for sustained Smad activation in response to prolonged BMP signals, leading to a feedforward loop where BMP enhances cytoskeletal motility and the motile cytoskeleton enhances BMP signaling. This exciting new mechanism opens the road to new investigations that might eventually establish a firm and potent role of cytoskeletal elements in the step-wise progression of the TGF-β signaling pathway.

11. Perspectives

In this article, we have highlighted specialized mechanisms that operate during molecular signaling by TGF-β family members. We have not covered all aspects of regulation of these pathways; rather we gave emphasis on areas of current and hopefully future active investigation. Such areas revolve around the still open problem of receptor and Smad trafficking and understanding the true biological meaning of such a complex process. Identification of novel regulators of trafficking is a major prerequisite together with the development of more sensitive cell biological tools that will allow the direct visualization of the dynamics of endogenous receptor and Smad movements in live cells and in real time. This should be coupled to approaches that examine the role of the cytoskeleton not only as a system that responds to the incoming TGF-β signals, but also as a system that provides signaling feedback to specific components of the pathway in order to cross-check its physiological flow.

The whole field of TGF-β signal transduction currently requires a thorough revisiting and clarification of the role of the various post-translational modifications that occur on either the receptors or the Smads. The emphasis must be on the timing of these events and on the characterization of sub-pools of the various protein complexes. The spectrum of post-translational modifications and interacting protein regulators of Smads and receptors will most certainly increase with time. The ultimate analysis of the network will additionally require sophisticated mathematical tools that might provide important predictions and aid in the elucidation of yet unknown regulatory mechanisms of this pathway. Finally, as the detailed knowledge of TGF-β signaling constantly increases, we should not forget that the TGF-β family includes many ligands. Not only is it important to elucidate the individual signaling details of each one of these – a task currently well underway [1] – but we need to remember that most cell systems are simultaneously or sequentially exposed to the action of several ligands of this family. Thus, measuring effects of TGF-β in an epithelial cell requires the consideration of the exact contribution of a parallel GDF pathway that is activated in the same cell and a slightly later coming BMP pathway. As these pathways share many of their signaling components, the integrated outcome of such combinatorial signaling can be difficult to predict. The current evolution of molecular and pharmacological tools for the dissection of all these pathways promises a thorough understanding of how cells interpret the interconnected signals from three or four TGF-β family ligands. We are optimistic that despite the firm establishment of this signaling pathway in textbooks, the next five years will offer significant revelations that will necessitate a serious rewriting of the central signaling network that gets mobilized every time a cell experiences ligands of the TGF-β family.

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